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Forced rotation of Na^+-driven flagellar motor in a coupling ion-free environment

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Abstract

Rotational characteristics of Na^+-driven flagellar motor in the presence and absence of coupling ion were analyzed by electrorotation method. The motor rotated spontaneously in the presence of Na^+, and the rotation accelerated or decelerated following the direction of the applied external torque. The spontaneous motor rotation was inhibited by removal of external Na^+, however, the motor could be forcibly rotated by relatively small external torque applied by the electrorotation apparatus. The observed characteristic of the motor was completely different from that of ATP-driven motor systems, which form rigor bond when their energy source, ATP, is absent. The internal resistance of the flagellar motor increased significantly when the coupling ion could not access the inside of the motor, suggesting that the interaction between the rotor and the stator is changed by the binding of the coupling ion to the internal sites of the motor.

Keywords: Flagellar motor; Coupling ion; Torque generation; Electrorotation

1. Introduction

Various membrane-linked systems, such as ATP synthesis, solute transports, and intracellular ion homeostasis, are energized by the flux of a certain ion driven by its electrochemical potential gradient. Significant insight into ion-coupling membranous systems has accumulated since the proposal of chemiosmotic theory by Mitchell. Nevertheless, very little is known about the exact molecular mechanisms of ion coupling and/or energy coupling in these systems. A bacterial flagellar motor is one of the molecular machines driven by the influx of H^+ (most neutrophiles) or Na^+ (alkalophiles and marine bacteria). Several candidate-subunits that play key roles in the torque generation and ion coupling of the motor have been suggested [1–4], and several types of theoretical models of torque-generating mechanisms have been proposed [5–8]. However, there is insufficient data to evaluate those models, such as the relationship between the rotation rate and ion flux.

Thus, to elucidate the molecular mechanism of torque generation in the flagellar motor, it should be necessary to further investigate the torque–rotation rate relationship in the environment with various concentration of coupling ion. Actually, the effect of internal and/or external coupling ion on the torque generation have been investigated for the Na^+-driven motor of *Vibrio alginolyticus* [9,10] and also for the H^+-driven motor of *Salmonella typhimurium* [11], and the essential role of the absolute concentration of coupling ions has been suggested. In the H^+-driven motor, however, it is difficult or almost impossible to largely change the concentration of coupling ion, since a change in H^+ concentration brings a change in the medium pH, which generally has serious effects on various cellular functions. Conversely, the concentration of coupling ion can be changed in the Na^+-driven motor without any difficulty, and removal of the Na^+ is also possible. Furthermore, specific inhibitors are also available for the motor [12,13].

The output of the flagellar motor is a mechanical rotation, and it can be artificially manipulated by the electrorotation technique [14–16] or by the optical tweezers [17]. Thus, these techniques are suitable for investigating the torque generation of the motor over a wide range of rotation rates in various environments. However, they have only been

Abbreviations: CCCP, carbonylcyanide-*m*-chlorophenylhydrazone; Tris, 2-amino-2-hydroxymethyl-1,3-propandiol; rps, revolutions per second

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applied to H^+-driven flagellar motors so far. In this paper, we applied the electrorotation technique to the tethered cell of an alkaliphilic bacterium, and investigated the rotational characteristics of the Na^+-driven flagellar motor in the environment with or without its coupling ion. The result showed that even in the absence of coupling ion, the motor never locked and could be forcibly rotated by applied torque much smaller than that spontaneously generated by the motor.

2. Materials and methods

2.1. Bacterial strain and growth condition

We used alkaliphilic bacterium AF112 that was isolated from pond mud in this study. A partial nucleotide sequence (441 bp) of 16S ribosomal RNA was determined and registered in DNA Data Bank of Japan (accession No. AB092342). The sequence showed 97% similarities to that of alkaliphilic *Bacillus* sp., DSM8715 [18]. The flagellar rotation of AF112 absolutely required Na^+ and was inhibited by phenamil, which is known as a specific inhibitor for Na^+-driven flagellar motors [12,13]. Thus, the flagellar motor of AF112 was concluded to be Na^+-driven type (Sugiyama et al., unpublished data). The cells were grown with shaking at 37 °C on AB4 medium consisting of 10 g polypepton, 1.5 g yeast extract, 10 g glucose, 1.5 g KH_2PO_4, 7.5 g Na_2CO_3 per litter (pH 9.5).

2.2. Tethered cell

At late logarithmic phase, an aliquot of the cells was withdrawn and dropped on a glass slide with electrodes. The cell suspension was covered with a glass cover slip with spacer and allowed to form tethered cells by physical adsorption of the flagellum to the glass surface. Then, the tethered cells were extensively washed with 10 mM Tris–HCl pH 9.5 supplemented with 10 mM NaCl or KCl and used for the electrorotation experiment. The concentration of contaminated Na^+ in the Na^+-free medium (10 mM Tris–HCl pH 9.5, 10 mM KCl) was determined to be 23 μM by atomic absorbance spectrophotometry (SAS7500A, Seiko Instruments).

2.3. Electrorotation experiment

The experimental apparatus for electrorotation and an example of the raw data was shown in Fig. 1. The system was essentially the same as that described in Ref. [14] except that glass slides with eight aluminum electrodes (Matsunami Glass Ind., Ltd.) and an eight-phase high frequency power supply (Shiba Denshi Systems Co., Ltd.) were used. A rotating electric field (500 kHz) was generated in the center space of the electrodes, which applied the external torque to tethered cells. The rotation rates of the tethered cell were determined from the intervals of light intensity peaks of the raw data.

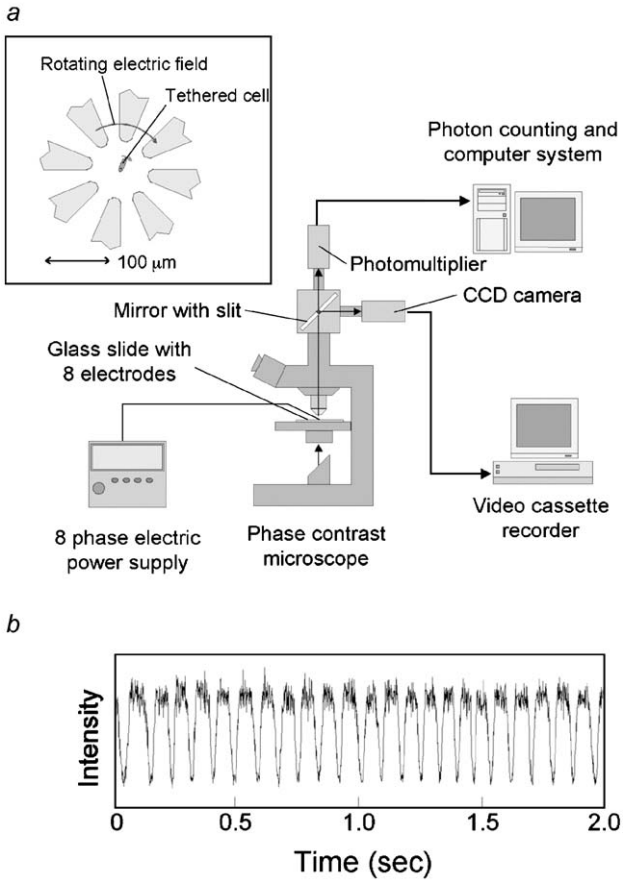

Fig. 1. Schematic drawing of an experimental apparatus and an example of raw data. (a) Schematic drawing of the apparatus for the tethered cell electrorotation experiments. An image of a rotating tethered cell was focused on a slit located at the center of a mirror. The light intensity passing through the slit was detected by the photon counting system. Insets: schematic drawing of the electrodes and a tethered cell on a glass slide. The diameter of the center space surrounded by the electrodes was ca. 60 μm. (b) An example of the raw data. The rotation rate was determined from the intervals of the light intensity peaks.

One series of the experiment was performed as follows: (1) the external torque was applied in 10 mM Tris–HCl (pH 9.5) containing 10 mM Na^+; (2) Na^+ was removed by washing with 10 mM Tris–HCl (pH 9.5) containing 10 mM K^+. Alternatively, the buffer was exchanged by 10 mM Tris–HCl (pH 9.5) containing 10 mM Na^+ and a protonophore, carbonylcyanide-*m*-chlorophenylhydrazone (CCCP), or a specific inhibitor, phenamil; (3) the torque was applied to the same cell again; and (4) the medium was exchanged with the original Na^+-containing buffer and the recovery of spontaneous rotation was observed. The final step was performed to confirm that the motor was not damaged during the Na^+ removal period.

2.4. Media and chemicals

Ultrapure water from Milli-Q A10 biocel system (Millipore Corp.) was used for preparing all media. Phenamil

was purchased from Molecular Probe Inc. Polypepton and yeast extract were purchased from Nihon Pharmaceutical Co. Ltd., and Difco Laboratories, respectively. All other chemicals were purchased from Wako Pure Chemical Ind., Ltd.

3. Results

The rotational characteristics of Na^+-driven flagellar motor of AF112 were investigated by the electrorotation method. The normalized rotation rates of AF112 tethered cells in the presence of 10 mM Na^+ are shown in Fig. 2 (open circles) as a function of the square of the applied voltage that is proportional to the externally applied torque [14]. When no electric field was applied, the cells spontaneously rotated at an average rotation rate of 2.8 revolutions per second (rps). The application of forward (the same direction as the spontaneous rotation of the tethered cell) external torque accelerated the rotation linearly. The rotation rate increased to twice the spontaneous rotation at 7.2 V (51.8 V^2) of applied voltage, indicating that the magnitude of the externally applied torque at that voltage corresponded to that of the spontaneous motor torque. Conversely, the motor rotation was decelerated by backward external torque. The average ratio of the rotation rate change to the square of the applied voltage ($\Delta\omega-V^2$ ratio) was 0.051 ± 0.002 rps/V^2.

In contrast, the flagellar motor no longer spontaneously rotated in the absence of Na^+. However, the motor could be forcibly rotated both forwards and backwards following the direction of the applied torque, as shown in Fig. 2 (closed circles). The $\Delta\omega-V^2$ ratio was 0.021 ± 0.003 rps/V^2, about 40% of that in the presence of Na^+, suggesting that the internal resistance of the motor is significantly increased in the absence of a coupling ion. It must be noted that we used only the data obtained by applying forward external torque in the following analyses and experiments, since the application of backward external torque occasionally caused unstable motor rotation (data not shown). Similar phenomena were also reported previously by Berry and Berg [19]. Furthermore, the process switching the direction of applied torque frequently caused a little change or displacement of the cell posture, which affected the size of external torque and generated slight difference of $\Delta\omega-V^2$ ratios between the directions. Actually, in some cases, a little difference in the slope of $\Delta\omega-V^2$ curves between forward and reverse directions was observed.

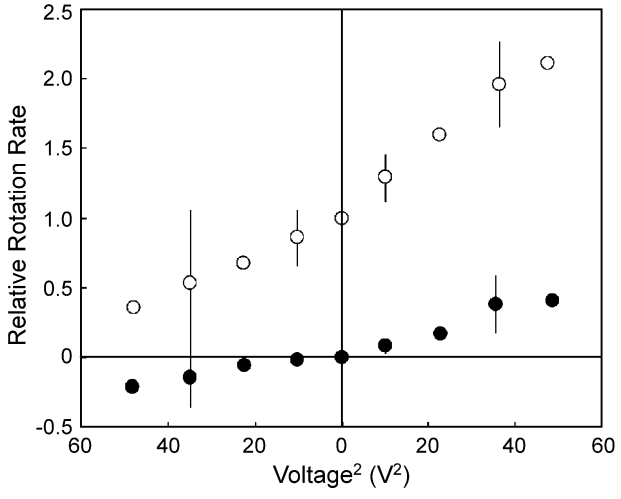

Fig. 2. Rotation rates of the tethered cells as a function of the applied external torque. The rotation rates of AF112 tethered cells in the presence (open circles) or absence (closed circles) of Na^+ were measured as a function of the square of the applied voltage. The data are indicated as values relative to the average spontaneous rotation rate of the tethered cells (2.8 rps). Each data point in the presence and absence of Na^+ was an average of eight and three independent experiments, respectively, using different tethered cells. The vertical lines on the data points at 10.2 and 35 V^2 in both directions indicate the standard deviation.

Table 1
Effect of buffer composition on $\Delta\omega-V^2$ ratio

Cell	Salt	$\Delta\omega-V^2$ ratio[a]
Tethered cells with broken motor[b]	NaCl (10 mM)	0.077 ± 0.017
	KCl (10 mM)	0.071 ± 0.022
Non-motile floating cells[c]	NaCl (10 mM)	0.58 ± 0.19
	KCl (10 mM)	0.65 ± 0.27

[a] The averages ± standard deviations measured for 10 tethered cells with broken motor and more than 100 floating cells were shown.

[b] Tethered cells that could not rotate in Na^+-containing buffer but could rotate when the rotating electric field was applied were judged to have broken motors.

[c] The rotation rates were determined from a video image of the rotating cells.

The lowest voltage in Fig. 2, 3.2 V (11.2 V^2), was sufficient to forcibly rotate the motor. The external torque applied at the voltage was estimated to be ca. 20% that of the spontaneously rotating motor in the presence of 10 mM Na^+ under the assumption that the external torque at 7.2 V (51.8 V^2) corresponded to the spontaneous torque of the motor. Forced rotation without Na^+ at much smaller external torques (at 1.9 V, corresponding to 7% of the spontaneous torque) was also observed (data not shown).

In electrorotation experiments, the same concentration of K^+ was added to the medium in the absence of Na^+ to maintain a constant ion strength. However, the torque acting on the tethered cell might be affected by the medium composition. To confirm this, a rotating electric field was applied to the non-motile floating cells and to the tethered cells with a broken motor, and their $\Delta\omega-V^2$ ratios were measured. No significant changes in the $\Delta\omega-V^2$ ratio due to the medium composition were observed in either case (Table 1).

We also analyzed the $\Delta\omega-V^2$ characteristics under conditions in which the motor could not rotate spontaneously but a coupling ion was present. These conditions were produced using CCCP or phenamil [12,13]. The typical results are provided in Fig. 3. The motor could be rotated by external torque even when the cell was de-energized by 100 μM CCCP in the presence of Na^+ (Fig. 3a). The $\Delta\omega-V^2$

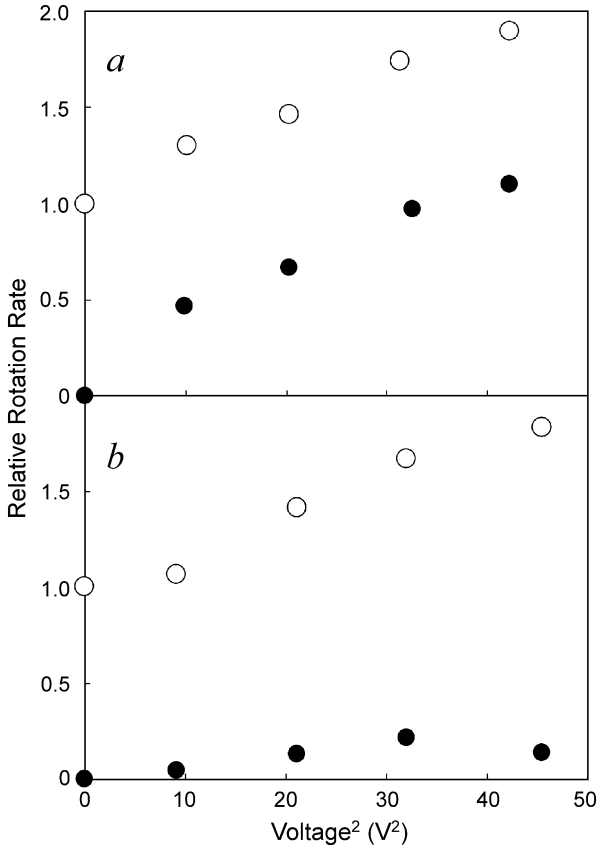

Fig. 3. The effects of CCCP and phenamil on the $\Delta\omega - V^2$ ratio of the motor. Relative rotation rate of AF112 tethered cells before (open circles) and after (closed circles) the addition of (a) 100 μM CCCP and (b) 100 μM phenamil are shown as a function of the square of the applied voltage. Spontaneous rotation rates of the tethered cells in (a) and (b) were 4.7 and 5.8 rps. The $\Delta\omega - V^2$ ratios for before and after the addition of the reagents were (a) 0.109 and 0.124 rps/V^2, and (b) 0.096 and 0.030 rps/V^2, respectively. Experiments were performed in 10 mM Tris pH 9.5 supplemented with 10 mM NaCl.

ratio of the de-energized cell relative to the spontaneously rotating one was 1.05, indicating that the internal resistance was almost the same as that in the absence of CCCP. In contrast, the motor for which rotation was inhibited by 100 μM phenamil exhibited a much smaller relative $\Delta\omega - V^2$ ratio (0.29), suggesting an increase in internal resistance (Fig. 3b).

4. Discussion

In this report, we demonstrated that applied external torque could forcibly rotate the Na^+-driven flagellar motor in a coupling ion-free environment. The result clearly shows that the motor is never locked even when the ion influx is absent, indicating that the motor rotation is not always tightly coupled with the ion influx. The absence of the tight coupling between the motor rotation and ion flux were also suggested from the experiments by Berry and Berg [17] using optical tweezers and H^+-driven motors, and also from the theoretical models of which rotational mechanisms are loosely coupled with ion influx as first suggested by Ref. [6].

In the absence of Na^+, the flagellar motor could be forcibly rotated with an external torque of only 7% (at 1.9 V applied voltage) of the spontaneously generated one. At this condition, the force required to slide a single force-generating unit against the rotor can be estimated to be 0.44 pN under the following assumption: the external torque of 7.2 V applied voltage is equal to the spontaneous motor torque in the presence of 10 mM Na^+, the spontaneous torque is 1.3×10^{-18} N m (calculated by assuming that the rotation rate of the tethered cell is 2.8 rps and the cell had ellipsoidal morphology with a polar radius of 1 μm and an equatorial radius of 0.5 μm), and the diameter of the rotor and the number of force-generating units are 40 nm and 10, respectively. The minimum required force is possibly much smaller than this value, since the tethered cell was forcibly rotated at 0.7 rps at the applied voltage, which was far from a stall. Thus quite small force was enough to forcibly slide the force-generating unit in the absence of energy source, i.e. influx of the coupling ion.

This characteristic of the flagellar motor was distinct from those of ATP-driven motor systems in eukaryotes, such as actin–myosin, tubulin–kinesin, tubulin–dynein. In the absence of ATP, the motor proteins of such systems are tightly associated with each other and form a rigor bond. The average unbinding force of a rigor bond between a single heavy meromyosin and an actin filament is ca. 9 pN that is two to five times larger than the sliding force [20]. However, in the case of flagellar motor, the required force to take place the sliding between the force-generating unit and the rotor without energy source is estimated to be less than 0.44 pN, which is much smaller than that of the actin–myosin motor system. Therefore, the energy transduction mechanism of the flagellar motor is suggested to be unique and completely different from those of ATP-driven motor proteins.

The present experiment also showed that Na^+ depletion causes significant increase in internal resistance of the motor. This was also the case when phenamil was added to the cells. Phenamil is considered to inhibit the rotation of Na^+-driven motor by blocking Na^+ influx but not to affect the sodium electrochemical potential of the cell [13]. Conversely, CCCP did not make any changes in the internal resistance. Since the respiration chain of alkaliphilic *Bacillus* has been known to extrude proton [21], the addition of excess CCCP completely collapses the proton electrochemical potential, i.e. the primary energy source, of the cell. Thus, the excess CCCP causes the disappearance of the Na^+ electrochemical potential generated by secondary Na^+/H^+ antiporter without influencing the accessibility of Na^+ to the motor. These results indicate that the interaction between the stator and rotor is relatively weak when the coupling ion can access the inside of the motor but becomes stronger when the ion access is prevented by the inhibitor or the removal of external Na^+.

The importance of absolute concentration of the coupling ion for the torque generation of the motor was suggested previously [9–11]. Sowa et al. [10] investigated the torque–rotation rate relationship in various concentrations of external Na^+ by detecting the position of a flagellar filament-attached bead by using the optical nanometry. They reported that the motor torque decreased with decreasing external concentration of Na^+, and suggested that the association and dissociation of coupling ion to the force-generating units can be the essential process for this phenomenon. In the present study, we showed that the internal resistance of the motor was significantly increased in the absence of coupling ion. This fact provide another factor for explaining the torque–rotation rate relationship of the motor in addition to the ion association–dissociation processes. By decreasing external Na^+ concentration, the empty duration of each Na^+ binding site becomes longer. This may strengthen the interaction between the rotor and the force-generating units depending on the site-empty period, which increase the internal resistance and decrease the generated torque of the motor.

Na^+-driven membranous systems of alkaliphilic bacteria, including the flagellar motor, play important roles in many cellular functions, such as swimming, active transport, intracellular pH and ion homeostasis [21,22]. However, their bioenergetics problems have not yet been fully solved [22]. The characteristic of Na^+-driven flagellar motors clarified in this study is expected to provide valuable insight for understanding Na^+-coupled bioenergetics in alkaliphilic bacteria, and also for investigating Na^+- and other ion-coupled membranous systems of various organisms.

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